Glycolytic enzymes and the control of glycolysis in dormant cysts of the brine shrimp Artemia salina.

Silvano Antonio. De Monte

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GLYCOLYTIC ENZYMES AND THE CONTROL OF GLYCOLYSIS IN DORMANT CYSTS OF THE BRINE SHRIMP ARTEMIA SALINA

by

Silvano Antonio De Monte

A Thesis submitted to the Faculty of Graduate Studies through the Department of Biology in Partial Fulfillment of the requirements for the Degree of Master of Science at The University of Windsor

Windsor, Ontario, Canada
1976
ABSTRACT

This study was conducted in an attempt to elucidate the mechanism of control of carbohydrate metabolism in encysted embryos of the brine shrimp, *Artemia salina*, exposed to anoxia for periods up to three months. Under anoxia, we observed that the lactic acid content of encysted embryos increased from 4.6 to 11.6 μg per mg cyst protein after one day, then remained constant for the next three months. The increase in lactic acid content in these embryos occurs at about the time control embryos (in air) begin to emerge from the chitinous shell. The constancy in lactic acid levels between days one and ninety is similar to that reported previously by other investigators. The presence of most glycolytic enzymes in encysted embryos was tested by measuring the ability of homogenate preparations to utilize glycolytic intermediates and cofactors in the production of lactic acid. The most active enzymes appeared to be aldolase, pyruvic kinase, and LDH. The least active glycolytic enzymes were found to be hexokinase, triosephosphate isomerase, and glyceraldehyde-3-phosphate dehydrogenase. The remaining glycolytic enzymes, trehalase, phosphoglucosemutase, phosphohexoisomerase, phosphofructokinase, phosphoglyceromutase, and enolase appear to be slightly active. However, when high concentrations of selected glycolytic intermediates and cofactors were added to a crude enzyme fraction (CEF) derived from cysts, lactic acid production was stimulated. Also, the activity of trehalase was increased twofold by treatment of CEF with 60% ethanol. The addition of glycolytic intermediates or cofactors alone to cyst homogenates did not stimulate the production of lactic acid except for NADH which may have allowed the conversion of endogenous pyruvate to lactate. In addition, no small
molecular weight inhibitor of glycolysis was found. From these results it appears that neither a lack of glycolytic enzymes nor a glycolytic pathway inhibitor can account for the low levels of lactic acid produced in Artemia salina cysts under anoxia and that low levels of glycolytic intermediates and/or limiting amounts of ATP and NAD may cause the metabolic block. The importance of ATP and NAD in the production of lactic acid by cyst homogenates and crude enzyme fraction was studied. Our results indicate that glucose utilization leading to the formation of lactic acid requires the addition of ATP and NAD⁺. These findings are consistent with our observation that encysted embryos of Artemia salina are deficient in NAD and low in ATP compared to nauplii. The results of this study suggest that in the absence of oxygen, Artemia salina cysts terminate carbohydrate metabolism due to the depletion of ATP and limiting supply of NAD.
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Grateful acknowledgement is extended to the following persons whose active participation have made this thesis possible.

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Last, but not least, I would like to acknowledge my parents, Mr. and Mrs. Giuseppe De Monte, who supported me while working on this thesis. May their hope of a better life for me be fulfilled.

My special thanks go to Ms. Sarah Jane Gilmore for typing this thesis.
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INTRODUCTION

The ability of embryos to develop or maintain viability in the absence of oxygen has been studied previously and reviewed by several authors (Barth and Barth, 1954; Brachet, 1960; Needham, 1963). It is apparent from the literature available that great variations in sensitivity to anoxia exist among eggs of different species. In general, however, the ability of embryos to grow anaerobically declines with age and although early embryonic development may be possible under anaerobiosis, it is eventually lost (Needham, 1963). For example, there is evidence that certain amphibian and trout zygotes will complete cleavage up to the blastula stage under anoxia; however they will not gastrulate and soon begin to cytolyze (Brachet, 1935; 1960; Needham, 1963). Cohen (1953) found that frog embryos maintained anaerobically accumulate lactic acid in all of the early stages of development, whereas only traces of lactic acid are found under normal aerobic conditions. Thus, the energy for early development in these embryos may be derived from anaerobic glycolysis. On the other hand, the eggs of Fundulus, a bottom fish, were observed to develop for 15 hours in the complete absence of oxygen and to remain viable for four days of anaerobiosis. In contrast, the eggs of the fish Ctenolabrus were killed after only four hours in carbon dioxide (Needham, 1963). The eggs of the sea urchin and Ascaris, a parasitic nematode, do not cleave under anoxia (Brachet, 1960). Yet, the development of Ascaris does not stop immediately and in the absence of oxygen it is able to form the chitinous envelope, the internal membrane and the perivitelline space, and eliminate the two polar
bodies (Needham, 1963). Among the crustaceans, most studies on the role of anoxia during development have been conducted on encysted embryos of the brine shrimp, *Artemia salina*, although some work has been done on *Simocephalus vetulus* (Reverberi, 1971). The present study on dormant cysts of *Artemia salina* was undertaken to further our understanding of control mechanisms during embryonic development.

The ability of encysted embryos of the brine shrimp to withstand long periods of anoxia is an interesting phenomenon. It was first reported by Dutrieu and Chrestia-Blanchine (1966) and later confirmed by other investigators (Ewing and Clegg, 1969; Stocco et al., 1972) that hydrated encysted embryos of *A. salina* can tolerate up to five months of anoxia without significant loss in developmental potential. Examination of the egg constituents by Dutrieu and Chrestia-Blanchine (1966) after two months of anoxia revealed that trehalose, glycerol, and total lipid levels increased slightly, whereas glycogen decreased slightly. Low levels of both lactic and pyruvic acids were also observed. These investigators concluded that metabolism in encysted embryos of *A. salina* under anoxia is extremely low or at least not very apparent. When the encysted embryos were returned to an aerobic environment, they developed into palaupluid and nauplii at the same rate (and extent) as embryos maintained in air only, notwithstanding a time delay in the onset of emergence of palaupluid. In an attempt to understand this unique response to anoxia, Ewing and Clegg (1969) analyzed developing embryos of *Artemia* for lactate dehydrogenase (LDH) levels and their ability to produce lactic acid in response to short periods of anaerobiosis. They
reported that the LDH activity is low in encysted embryos and remains low during pre-emergence development. During later development (emergence and hatching stages) the LDH level in the embryo increased markedly. The increase in LDH activity at emergence correlated well with the increased production of lactic acid in response to anoxia. These investigators suggested that low levels of LDH in encysted embryos may be important in the regulation of carbohydrate metabolism during periods of anoxia. In contrast to these findings on carbohydrate metabolism, studies by Stocco et al. (1972) demonstrated that nucleotide metabolism in *A. salina* cysts continues during anoxia and pronounced changes occur in the free nucleotide pools. The results of this latter study are significant to the investigation reported here in view of the fact that the adenosine nucleotide content (ATP, ADP) of *A. salina* cysts was reported to fall markedly during the first few days of anoxia and becomes undetectable after about a week under nitrogen. However, upon aeration, and following a short time lag, the adenosine nucleotide levels return to those found in embryos incubated in air only. From these studies, it appears that in the absence of oxygen, hydrated encysted embryos of *A. salina* permit nucleotide metabolism but not carbohydrate metabolism.

This thesis attempts to elucidate the inability of dormant cysts of *Artemia salina* to carry on carbohydrate metabolism under anoxia. To this end, the level of lactic acid in encysted *Artemia* embryos maintained under anoxia for periods of up to three months was studied. Also, the level of lactic acid in the incubation medium of those embryos maintained under anoxia was measured. The activities of the glycolytic enzymes in homogenates or partially purified protein fractions from
undeveloped cysts were also measured. Finally, the importance of the nucleotide and cofactor requirements for glycolysis was investigated and the level of these components in undeveloped and developed embryos determined. The results of these studies are presented in this thesis and discussed as they relate to the control of carbohydrate metabolism and development in encysted embryos of *Artemia salina* under anoxia.
MATERIALS AND METHODS

Preparation of Artemia Cysts for Long Term Incubation Under Nitrogen

Dry cysts of Artemia salina were obtained from Canadian Aquarium Supplies Co. Ltd. (St. Thomas, Ontario). Initially one batch of cysts was incubated aerobically for 6 hours at 30°C in artificial sea water (ASW) (Warner and McClean, 1968) while a second batch of cysts was maintained aerobically at 0°C for 6 hours in ASW. Following these treatments the cysts were collected separately on sintered glass filters and suspended in 7% antiformin for 15 - 30 minutes at 0 - 5°C as previously described (Nakanishi et al., 1962). All floating cysts and debris of each batch were removed by suction and the remaining cysts were washed on a sintered glass filter with one liter of 10% urea and then distilled water. Six-gram quantities (wet weight) were placed in sterile (autoclaved at 121°C for 15 minutes) 50 ml glass ampules (Wheaton Glass Co., South Brampton, Ontario) and covered with 50 ml of sterile ASW fortified with 1000 units/ml of penicillin G (Squibb or Ayerst) and 100 µg/ml of streptomycin sulfate (Mann Research Laboratories) (Warner and McClean, 1968). The ampules were purged aseptically for 30 minutes with purified nitrogen (Stocco et al., 1972) then heat sealed and stored at room temperature until needed.

1To remove residual amounts of hypochlorite from the antiformin treatment.
Periodically, the ampules were checked visually for air leaks and microbial contamination. After various periods of anoxia (up to 3 months) the ampules were opened and the cysts collected on a sintered glass filter and washed with distilled water. In addition, the incubation medium was collected and filtered again through paper (Whatman #1). Both cysts and medium were stored at -15°C until needed.

**Preparation of Artemia Cysts for Short-Term Incubation Under Nitrogen**

Dry cysts were treated for 15 - 30 minutes with 7% antiformin and washed thoroughly with urea and distilled water as described above. Five grams cysts (wet weight) were placed into each of three flasks containing 100 ml of ASW fortified with penicillin G and streptomycin sulfate as above. All flasks were kept at 0°C while one flask was purged with purified nitrogen and another aerated vigorously. The third flask was untreated and maintained at 0°C as a control. After 30 minutes the nitrogen treated and aerated flasks were placed in a shaking water bath (Dubnoff) at 30°C and the treatments continued for the duration of the experiment. At selected times (up to 27 hours) 5.0 ml aliquots were removed from each of the three flasks and filtered through filter paper (Whatman #1) to collect the cysts. A small quantity of cysts (0.1 gram wet weight) from each sample was used directly for the determination of lactic acid and protein content as described below. Also, one-half of the contents remaining in the aerated flask after 24 hours of incubation at 30°C was transferred to another flask and purged with
nitrogen at 30°C for an additional 3 hours. At the end of this period, the contents of both flasks (aerated and nitrogen-purged) were filtered through filter paper as above.

Determination of Lactic Acid Content in Artemia Cysts and Incubation Medium

The lactic acid content of cysts and incubation media was determined by the method of Barker (1957) as follows. A sample of cysts (usually 0.1 gram wet weight) was homogenized in a glass homogenizer (Duall type) with 10.0 ml of ice-cold buffer A (KHCO₃ 0.025 M; MgCl₂, 0.004 M; KCl, 0.015 M; KH₂PO₄, 0.02 M, adjusted to pH 7.5 with KOH) and 2.0 ml 20% TCA similar to that described by Gregg et al. (1964) for the measurement of lactic acid in homogenates of amphibian eggs. The homogenate was centrifuged at 15,000 g for 15 minutes and 5.0 ml of the supernatant fluid was added to 5.0 ml of 4% CuSO₄·5H₂O. To this solution was added 1.0 gram of Ca(OH)₂ and the mixture shaken vigorously then allowed to stand for 30 minutes. The insoluble material was removed by centrifugation and duplicate 1.0 ml aliquots of supernatant fluid were pipetted into screw capped tubes containing about 0.05 ml (1 drop) of 4% CuSO₄·5H₂O. Six ml of concentrated sulfuric acid was added to each tube at 0°C and the tubes capped and immediately shaken. The tubes were then placed in a boiling water bath for 5 minutes. After cooling, two drops (0.1 ml) of 1.5% phenylphenol were added to each tube and mixed thoroughly. The tubes were incubated at 30°C for 30 minutes, then placed in a boiling water bath for 90 seconds. After cooling, the absorbance at 560 nm was read in a
Beckman spectrophotometer (Model DB). The lactic acid content of the incubation medium was also measured using this procedure. The amount of lactic acid in both cysts and medium was determined using appropriate amounts of lithium lactate in companion reactions as standards. Protein was determined by the method of Lowry et al. (1951) following delipidation of the homogenate according to the procedure of Bligh and Dyer (1959).

Preparation of Crude Enzyme Fraction (CEF) from Artemia Cysts

Dry cysts were hydrated and washed extensively as described above. The washed cysts were homogenized in about 5 volumes of ice-cold buffer A containing 1 mM dithiothreitol (Calbiochem) and the homogenate was centrifuged at 15,000 g for 15 minutes. Unless otherwise indicated all steps were carried out at 0 - 5°C. The supernatant fluid was removed with a pipette and concentrated by vacuum dialysis to approximately one-tenth of the original volume. The protein concentrate was dialyzed further for 12 hours against two 500 ml changes of buffer A containing 1 mM DTT, and then stored at -15°C. This crude enzyme fraction (CEF) was used to test for glycolytic enzyme activities.

The dialyzate from the vacuum dialysis step was concentrated at reduced pressure in a rotavapor (Büchi) and any insoluble material was removed by centrifugation. The dialyzate was then desalted by passage through a column of Sephadex G-10 (1 x 55 cm) using water as the eluant. The UV absorbing material which eluted in the void volume was concentrated by flash evaporation and stored at -15°C.

The protein content of the CEF was determined as indicated above.
Determination of Trehalase Activity in the CEF of Artemia Embryos

Trehalase activity was determined on both CEF and ethanol-treated CEF of Artemia cysts (Ewing, personal communication). A sample of CEF was divided into two equal parts. One part was adjusted to 5.0 ml with Buffer A and kept at 0°C, while the other part was treated with ethanol to a final concentration of 60%. The ethanol-treated CEF was stirred for 15 minutes at 0°C and the precipitate collected by centrifugation. The ethanol precipitate was resuspended in Buffer A and adjusted to a final volume of 5.0 ml as for the CEF above. The hydrolysis of trehalose was measured according to the method of Hill and Sussman (1963) slightly modified. The reaction mixtures contained 1.5 ml of either CEF or ethanol-treated CEF and varying concentrations of trehalose in Buffer A in a final volume of 10 ml. All reaction vessels were incubated at 30°C. At various times, 2.0 ml aliquots were removed and added to 2.0 ml of distilled water at 80°C, then maintained at 100°C for 4 minutes to stop the reaction. The precipitate was removed from the heat-treated samples by centrifugation and an aliquot of the supernatant fluid was removed for the colorimetric determination (540 nm, Clark, 1964) of reducing sugar (as glucose) by the method of Somogyi (1952) using Nelson's reagent.
Glycolytic Enzyme Activities in Artemia Cyst Homogenates

Glycolytic enzyme activities were determined by measuring lactic acid production in Artemia cyst enzyme preparations (homogenates, post-mitochondrial supernatants, or CEP) incubated with various glycolytic intermediates as substrates. Therefore, the activity of each glycolytic enzyme was determined by adding the appropriate substrate and cofactor(s) to standard reaction mixtures containing a cyst enzyme preparation in Buffer A. The concentrations of substrate and cofactor(s) used in each experiment varied; therefore they are indicated in the Tables and Figures. The reaction was started by the addition of a pre-determined amount of cyst enzyme preparation to the reaction vessel. The reaction vessels were incubated at 30°C and aliquots were removed at 0, 10, 20 and 30 minutes for the determination of lactic acid content as described above.

Most incubations were carried out in air, since early experiments under nitrogen gave similar results for the enzymes aldolase and lactate dehydrogenase (LDH).

Using the above procedure the following enzymes were assayed: trehalase (3.2.1.28), phosphoglucomutase (2.7.5.1), hexokinase (2.7.1.1), phosphohexoisomerase (5.3.1.9), phosphofructokinase (2.7.1.11), aldolase (4.1.2.13), triosephosphate isomerase (5.3.1.1), glyceraldehyde-3-phosphate dehydrogenase (1.2.1.12), phosphoglyceromutase (5.4.2.1), enolase (4.2.1.11), pyruvic kinase (2.7.1.40), LDH (1.1.1.27), and L-α-glycerophosphate dehydrogenase (1.1.1.8).

Substrates and cofactors were purchased from the following commercial sources: trehalose from Nutritional Biochemicals Corporation; glucose from
Fisher Scientific Co.; glucose-1-phosphate\textsuperscript{2} and glucose-6-phosphate from Mann Research Laboratories; and fructose-1,6-diphosphate, dihydroxyacetone phosphate, L-\(\alpha\)-glycerophosphate, glyceralsedehyde-3-phosphate, phosphoenol pyruvate, and pyruvic acid from Sigma Chemical Co. The nucleotides NAD\textsuperscript{+}, NADH, ADP, ATP were purchased from both Mann Research Laboratories and Sigma Chemical Company.

Determination of Nicotinamide Adenine Dinucleotide Levels in Artemia Cysts and Nauplii

a) Total NAD\textsuperscript{+} and NADH Content

The concentration of total nicotinamide adenine dinucleotide levels (NAD\textsuperscript{+} plus NADH) in Artemia cysts and nauplii was determined on cold acid extracts of cysts or nauplii as follows. Ten grams of dry cysts or 2.0 grams of nauplii (wet weight) were homogenized in 7 - 10 volumes of ice-cold 1 N HClO\textsubscript{4}. The homogenates were centrifuged at 15,000 g for 15 minutes and the acid soluble material removed. The insoluble pellets were resuspended in small volumes of ice-cold 0.5 N HClO\textsubscript{4} and the acid soluble fractions collected as above. The pooled acid soluble extracts from cysts or nauplii were deacidified with alamine (Warner and Pianamore, 1967), then applied to separate columns of DEAE-cellulose (Whatman DE-23) (3 x 40 cm and 1 x 40 cm for cyst and nauplius preparations, respectively) and the nucleotides eluted with a linear gradient of NH\textsubscript{4}HCO\textsubscript{3}, pH 8.6, as previously.

\textsuperscript{2} The following abbreviations were used in this study: glc-1-P, glucose-1-phosphate; glc-6-P, glucose-6-phosphate; fru-6-P, fructose-6-phosphate; fru-1,6-P\textsubscript{2}, fructose-1,6-diphosphate.
described (Warner and Finamore, 1967). In a control experiment it was found that NAD⁺ elutes from DEAE-cellulose between guanosine and CMP. Therefore the contents of all the column fractions between the guanosine and CMP fractions were pooled and concentrated by flash evaporation to about 1.5 ml. The concentrations of NAD⁺ in these fractions were determined using the alcohol dehydrogenase (ADH) (1.1.1.1) method of Ciotti and Kaplan (1963) slightly modified. The reaction mixtures contained the following components in 1.5 ml total volume: 0.42 M ethanol, 0.083 M Na₂HPO₄·10H₂O, 25 units of ADH (Sigma Chemical Co.), 38 μg BSA, and 0.167 mM KH₂PO₄, adjusted to pH 7.5; and varying amounts of a standard NAD⁺ solution or DEAE-cellulose fraction as described above. The reaction mixtures were incubated in a jacketed cuvette and the reaction started by the addition of ADH. The production of NADH was measured spectrophotometrically at 340 nm and the amount of NAD⁺ in the extract was calculated from a standard curve using known amounts of NAD⁺.

b) NADH Content of Cysts and Nauplii

The relative content of NADH in cysts and nauplii homogenates was estimated using the endogenous LDH which requires NADH as a cofactor. Hydrated cysts or nauplii were homogenized in 10 volumes of Buffer A and the post-mitochondrial supernatant fractions were assayed for the production of lactic acid at various times after the addition of pyruvate (3.2 mM) and/or NADH (0.67 mM) to standard reaction mixtures. The reactants were incubated at 30°C and aliquots were removed at selected time intervals and treated with a one-fifth volume of 20% TCA. The fractions were centrifuged and lactic acid was measured in
the supernatant fluid as indicated above. The protein content of each enzyme preparation was also estimated as described above.
RESULTS

Lactic Acid Content in Encysted Artemia Embryos Maintained Under Nitrogen for up to Three Months

The effect of extended periods of anoxia on the production of lactic acid in undeveloped cysts and 6-hour embryos of Artemia salina was studied. When fully hydrated undeveloped Artemia cysts or 6-hour embryos are maintained in an anoxic environment in ASW at room temperature, they produced very little lactic acid after the first day to three months of anoxia. The results of this study are shown in Figure 1. Initially, the undeveloped cysts contain 4.6 ± 0.5 μg lactic acid per mg protein, whereas the 6-hour embryos contain 6.7 ± 0.1 μg lactic acid per mg protein. The difference between the two controls may be significant and suggests that lactic acid production occurs in embryos (at least to some extent) incubated in air at 30°C. During the first 24 hours of anoxia, however, the lactic acid content of both groups of embryos nearly doubled. Following this initial rise, the lactic acid levels in both populations of embryos remained relatively constant over the three month period and averaged 11.7 ± 0.9 and 12.6 ± 0.8 μg lactic acid per mg protein for 0-hour and 6-hour embryos, respectively. Furthermore, during incubation under anoxia very little lactic acid was lost to the medium from either population of cysts.

Lactic Acid Content in Encysted Artemia Embryos Maintained Under Nitrogen for up to 27 Hours

To determine the time of onset of lactic acid production in
The effect of anoxia on the level of lactic acid in undeveloped and 6-hour embryos of *A. salina*.

The embryos were maintained under nitrogen in sealed glass ampules at 22 - 24°C after nitrogen treatment. At the times indicated one ampule from each group of cysts was opened; and the cysts and incubation medium collected and processed for lactic acid determination as described under Materials and Methods.

- - - o, lactic acid levels in undeveloped cysts and medium;
- - - o, lactic acid levels in 6-hour embryos and medium.
Artemia embryos under anoxia, one batch of cysts was purged continuously for over 24 hours with purified nitrogen, and samples were removed periodically for lactic acid determination. Another batch of cysts was aerated continuously and sampled as above for lactic acid determination. The results of this study are shown in Figure 2. The data indicate that lactic acid production begins almost immediately (within 4 hours) in Artemia embryos whether incubated in air or nitrogen. However, the lactic acid levels in nitrogen-treated embryos were found to be consistently higher than in aerated embryos, although during the first 14 to 16 hours of treatment the levels closely resembled one another in both populations of embryos. Beyond this period of time the nitrogen-treated embryos exhibited a sharp increase in lactic acid content compared to the aerated embryos in which the lactic acid levels decreased. When 24-hour embryos from the aerated flask were purged with nitrogen for 3 hours, considerable lactic acid was produced. It appears, then, that lactic acid products in Artemia cysts in response to anoxia occurs mainly after the first 14 hours of anoxia at a time when emergence of the prenauplius larvae in air was first observed.

Glycolytic Enzyme Activities in Homogenates of Encysted Artemia Embryos

Thus far the results obtained in this study have shown that the hydrated dormant cysts of Artemia produce and/or accumulate only a small amount of lactic acid during incubation in nitrogen-purged ASW for up to three months. A similar response was obtained when homogenates of Artemia cysts were incubated either aerobically or.
The lactic acid content of *Artemia* embryos aerated or nitrogen-treated for up to 27 hours.

o—o, cysts kept at 0°C;
 o—o, cysts treated with nitrogen;
 o---o, cysts treated with air.

The arrow indicates the time at which prenauplii were first observed in the aerated flask.
anaerobically for various periods of time. Therefore, it appeared that one or more enzymes of the glycolytic pathway may be inactive in Artemia cysts or that limiting amounts of substrate(s) and/or cofactor(s) are present in the undeveloped cysts. When Artemia cyst homogenates were incubated with various intermediates of glycolysis, the results shown in Table 1 were obtained. The most active enzymes appear to be aldolase, pyruvic kinase, and lactic dehydrogenase (LDH). The least active enzymes are hexokinase, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, and L-α-glycerophosphate dehydrogenase. The remaining glycolytic enzymes, trehalase, phosphoglucomutase, phosphohexoisomerase, phosphofructokinase, phosphoglyceromutase, and enolase are only slightly active. Phosphoglycerokinase was not studied. Except for fru-1,6-P₂, the addition of substrates alone did not appear to activate any of the enzymes. The reason for the apparent enzyme activation by fru-1,6-P₂ is discussed below.

When Artemia cyst homogenates were incubated with various combinations of cofactors only the results shown in Table 2 were obtained. Although considerable variability occurs in the data, it appears that NADH alone (in the absence of added substrates) is the most important cofactor. Perhaps NADH is lacking or in extremely low quantities in the cyst homogenate and thus insufficient for the conversion of endogenous pyruvate to lactic acid by LDH in the final step of glycolysis.

The effect of nitrogen atmosphere on aldolase and LDH activities in cyst homogenates was also examined. The results are summarized in Table 3 and indicate that both enzymes function very well in an
Table 1. A comparison of glycolytic enzyme activities in Artemia cyst homogenates.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate (mM)</th>
<th>Cofactors (mM)</th>
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<td>Control</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trehalase</td>
<td>Trehalose</td>
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<td>0.15</td>
<td>0.15</td>
<td>0.067</td>
</tr>
<tr>
<td>Hexokinase</td>
<td>Glucose</td>
<td>0.32</td>
<td>0.15</td>
<td>0.15</td>
<td>0.067</td>
</tr>
<tr>
<td>Phosphoglucomutase</td>
<td>Glucose-1-phosphate</td>
<td>0.32</td>
<td>0.15</td>
<td>0.15</td>
<td>0.067</td>
</tr>
<tr>
<td>Phosphohexoisomerase</td>
<td>Glucose-6-phosphate</td>
<td>0.32</td>
<td>0.15</td>
<td>0.15</td>
<td>0.067</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>Fructose-6-phosphate</td>
<td>0.32</td>
<td>0.15</td>
<td>0.15</td>
<td>0.067</td>
</tr>
<tr>
<td>Aldolase</td>
<td>Fructose-1,6-diphosphate</td>
<td>0.32</td>
<td>0.15</td>
<td>0.15</td>
<td>0.067</td>
</tr>
<tr>
<td>Triosephosphate Isomerase</td>
<td>Dihydroxyacetone Phosphate</td>
<td>0.32</td>
<td>0.15</td>
<td>0.067</td>
<td></td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate</td>
<td>Glyceraldehyde-3-phosphate</td>
<td>0.32</td>
<td></td>
<td></td>
<td>0.067</td>
</tr>
<tr>
<td>Phosphoglyceromutase</td>
<td>3-phosphoglyceric acid</td>
<td>0.32</td>
<td>0.15</td>
<td>0.067</td>
<td></td>
</tr>
<tr>
<td>Enolase</td>
<td>2-phosphoglyceric acid</td>
<td>0.32</td>
<td></td>
<td></td>
<td>0.067</td>
</tr>
<tr>
<td>Pyruvic Kinase</td>
<td>Phosphoenolpyruvic acid</td>
<td>0.32</td>
<td>0.15</td>
<td>0.15</td>
<td>0.067</td>
</tr>
<tr>
<td>Lactate Dehydrogenase</td>
<td>Pyruvic acid</td>
<td>0.32</td>
<td></td>
<td></td>
<td>0.067</td>
</tr>
<tr>
<td>L-α-Glycerophosphate</td>
<td>L-α-glycerophosphate</td>
<td>0.32</td>
<td>0.15</td>
<td>0.15</td>
<td>0.067</td>
</tr>
</tbody>
</table>

All reaction mixtures contained 71 mg cyst homogenate protein in a buffer containing KHCO₃, 25 mM; KCl, 90 mM; KH₂PO₄, 20 mM; MgCl₂, 0.4 mM; and MgSO₄, 0.0 mM at pH 7.5, and the substrates and cofactors as indicated in 50 ml final volume. All reaction vessels were incubated at 30°C and aliquots were removed at various times for the determination of lactic acid as described in Materials and Methods.

a The standard deviation of the mean was based on n=10.

b The standard deviation of the mean was based on n=7.
Table 2. The effect of added cofactors on lactic acid production in *Artemia* cyst homogenates.

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Cofactors (mM)</th>
<th>µg lactic acid/ (mg protein/ hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP 0.15mM</td>
<td>0.6 ± 0.9^b</td>
</tr>
<tr>
<td>1</td>
<td>ADP 0.15mM</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>NAD⁺ 0.067mM</td>
<td>-0.2</td>
</tr>
<tr>
<td>3</td>
<td>NADH 0.067mM</td>
<td>1.4</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>1.6</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>1.3</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>0.7</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>-0.7</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>1.13</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>2.06</td>
</tr>
</tbody>
</table>

^a All reaction mixtures contained 71 mg cyst homogenate protein in Buffer A and cofactor(s) as indicated in 50 ml final volume. All reaction vessels were incubated at 30°C and aliquots were removed at various times for the determination of lactic acid as described in Materials and Methods.

^b The standard deviation was based on n=7.
Table 3. Lactic acid production by *Artemia* cyst homogenates incubated in air or nitrogen\(^a\).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate (mM)</th>
<th>Cofactors (mM)</th>
<th>Enzyme activity (µg lactic acid/ mg protein/ hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ADP</td>
<td>NAD(^+)</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDH (air)</td>
<td>pyruvate (0.32)</td>
<td>0.067</td>
<td></td>
</tr>
<tr>
<td>LDH (N(_2))</td>
<td>pyruvate (0.32)</td>
<td>0.067</td>
<td></td>
</tr>
<tr>
<td>Aldolase (air)</td>
<td>fru-1,6-P(_2) (0.32)</td>
<td>0.15</td>
<td>0.067</td>
</tr>
<tr>
<td>Aldolase (N(_2))</td>
<td>fru-1,6-P(_2) (0.32)</td>
<td>0.15</td>
<td>0.067</td>
</tr>
</tbody>
</table>

\(^a\)The only substrates tested were pyruvate and fru-1,6-P\(_2\) and the reaction conditions were as described in Table 2.

\(^b\)The standard deviation was based on \(n=4\).
anaerobic environment. The differences in the enzyme activities between the two treatments may be due to batch differences, since these experiments were done on different days.

Although some enzymes appear to be only slightly active in cyst homogenates, these results suggest that *Artemia salina* cysts contain all glycolytic enzymes required for the metabolism of trehalose to lactic acid under anoxia. The reason that intact encysted embryos fail to accumulate significant levels of lactic acid under anoxia may be due to either low levels of substrate and/or limiting amounts of cofactors (ATP, ADP, NAD).

The Activity of Trehalase in *Artemia* Dormant Cysts

Trehalose is the most abundant carbohydrate in the encysted embryo of the brine shrimp, *Artemia salina*, and accumulates to approximately 15% of the total cyst weight in embryos entering dormancy (Clegg, 1965). Studies by Clegg (1964) showed that in the dormant embryo trehalose is the respiratory substrate and most of it is converted to glycogen and glycerol during pre-emergence development. Since these observations suggested the presence of trehalase in *Artemia salina* embryos, it was interesting that the addition of trehalose did not stimulate the production of lactic acid in cyst homogenates (Table 1). Therefore, the activity of trehalase was tested in the CEF of cysts by the method of Hill and Sussman (1963). The results of this study are summarized in Table 4, and indicate the presence of trehalase in CEF of *A. salina* dormant embryos. However, it should be noted that the alcohol-treated CEF contains approximately twice the activity of the untreated CEF. It appears that trehalase in *A. salina* cysts may not be fully functional and may require activation (by some mechanism) for expression of its full
Table 4. Trehalase activity in crude enzyme fraction (CEF).²

<table>
<thead>
<tr>
<th>CEF preparation</th>
<th>Trehalose (mM)</th>
<th>µg glucose/hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.3</td>
<td>57.8</td>
</tr>
<tr>
<td>Ethanol-treated</td>
<td>5.3</td>
<td>114.4</td>
</tr>
</tbody>
</table>

²Trehalase activity was determined by measuring the appearance of glucose as a function of time in the presence of trehalose as indicated in Materials and Methods. The reaction vessel contained either CEF (3.1 mg protein) or ethanol-precipitated CEF in Buffer A. All reaction vessels were incubated at 30°C.
activity.

Activity of Selected Glycolytic Enzymes in a Crude Enzyme Fraction (CEF) of the Artemia Cyts

In the previous study of the glycolytic enzymes in cyst homogenates, it was observed that certain enzymes were present in very low quantities and therefore not very active in homogenates. Therefore, a crude enzyme fraction (CEF) was prepared from a cyst homogenate and tested for glycolytic enzyme activities using glucose, \text{glc}-6-P, fru-1-P, and fru-1,6-\text{P}_2 as substrates. The results of this study are shown in Table 5 and demonstrate that high concentrations of both substrate and cofactors are sometimes necessary for the detection of certain glycolytic enzymes. In particular, hexokinase and phosphofructokinase require high concentrations of substrate for activation compared to aldolase and phosphohexoisomerase. These results support further the argument that these glycolytic enzymes are present in dormant embryos of \textit{Artemia salina}, and suggest that the concentration of substrate and cofactors may be important in the activation of certain glycolytic enzymes.

The Effect of Small Molecular Weight Components from Artemia Cyts on Lactic Acid Production

To determine whether the small molecular weight components from the cysts were affecting glycolysis, this fraction (dialylicate) was obtained as described in Materials and Methods and added to the reaction mixtures containing CEF with either glucose or fru-6-P. The results of this study are presented in Figure 3. The data indicate that increasing
Table 5. Activity of selected glycolytic enzymes in CEF from *Artemia* cysts.a

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>[ATP]</th>
<th>[ADP]</th>
<th>[NAD+]</th>
<th>Enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mM)</td>
<td>(mM)</td>
<td>(mM)</td>
<td>(mM)</td>
<td>(µg lactic acid/mg protein/hr)</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-0.4</td>
</tr>
<tr>
<td>Hexokinase</td>
<td>Glucose</td>
<td>0.15</td>
<td>-</td>
<td>0.067</td>
<td>-1.0</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>0.75</td>
<td>-</td>
<td>0.34</td>
<td>7.0</td>
</tr>
<tr>
<td>Phosphohexoisomerase</td>
<td>Glc-6-P</td>
<td>0.15</td>
<td>-</td>
<td>0.067</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>Glc-6-P</td>
<td>0.75</td>
<td>-</td>
<td>0.34</td>
<td>11.9</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>Fru-6-P</td>
<td>0.15</td>
<td>-</td>
<td>0.067</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Fru-6-P</td>
<td>0.75</td>
<td>-</td>
<td>0.34</td>
<td>11.1</td>
</tr>
<tr>
<td>Aldolase</td>
<td>Fru-1,6-P₂</td>
<td>-</td>
<td>0.15</td>
<td>0.067</td>
<td>13.6</td>
</tr>
<tr>
<td></td>
<td>Fru-1,6-P₂</td>
<td>-</td>
<td>0.75</td>
<td>0.34</td>
<td>19.7</td>
</tr>
</tbody>
</table>

aThe reaction conditions were as in Table 2. The reaction mixture contained substrates and cofactors as indicated, and 13.8-18.2 mg CEF protein from dormant cysts in Buffer A in a final volume of 10 ml. All reaction vessels were incubated at 30°C and aliquots were removed at various times for the determination of lactic acid as described in Materials and Methods.
The effect of *Artemia* cyst small molecular weight fraction on lactic acid production by crude enzyme fraction (CEF).

(a) **Glucose as substrate.**

All reaction mixtures contained 5.1 mg CEF protein in Buffer A, 3.2 mM glucose, 1.5 mM ATP, 0.67 mM NAD⁺ plus no addition of dialyze (●—●), 26 A₂₆₀ units dialyze (●—●), or 52 A₂₆₀ units dialyze (●—●) in a final volume of 10 ml.

(b) **Fructose-6-phosphate as substrate.**

All reaction mixtures contained 2.8 mg CEF protein in Buffer A and 3.2 mM fru-6-P, 1.5 mM ATP, 0.67 mM NAD⁺ plus no addition of dialyze (●—●), 2.5 A₂₆₀ units dialyze (●—●), 10 A₂₆₀ units dialyze (●—●), or 50 A₂₆₀ units dialyze (●—●) in a final volume of 10 ml.

All reaction vessels were incubated at 30°C and aliquots were removed for lactic acid measurements at the times indicated.
amounts of dialyzate stimulate the production of lactic acid when added to reaction vessels containing either glucose or fru-6-P as the primary substrate and the necessary cofactors. The stimulation may be due to the addition of certain glycolytic intermediates or alternate energy sources for lactic acid production. In any case it is clear that the small molecular weight components of the cysts are not inhibitory to glycolysis.

The Effect of ATP and NAD⁺ on Lactic Acid Production by Artemia Cyst Post-Mitochondrial Fraction.

The ATP concentration in dormant brine shrimp cysts has been found to be relatively low compared to other nucleotides (Warner and Finamore, 1967; Stocco et al., 1972). In addition, the presence of NAD in brine shrimp cysts was previously reported (Warner and Finamore, 1965) although its concentration was not studied. Thus, an inadequate supply of ATP and/or NAD may be responsible for the low activity of cyst homogenates in lactic acid production. If glucose is added to cyst post-mitochondrial supernatant fluid, very little lactic acid is produced. However, if both glucose and ATP or NAD⁺ are added, lactic acid production is stimulated. The results of these experiments are shown in Table 6. The addition of ATP to the post-mitochondrial fraction supplemented with glucose appears to induce the production of more lactic acid than when added in combination with NAD⁺. The reason for this finding is not known, but it may be due to the accumulation of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, which have been found to interfere with the lactic acid assay. In the presence of NAD⁺ these glycolytic intermediates would be converted to other
Table 6. The effect of ATP and NAD\textsuperscript{+} on lactic acid production by the post-mitochondrial fraction of *Artemia* cysts\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Substrate added (mM)</th>
<th>Cofactors (mM)</th>
<th>Enzyme activity ((\mu g) lactic acid/mg protein/hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose 3.2</td>
<td>-</td>
<td>ATP 6.1</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>NAD\textsuperscript{+} 2.9</td>
</tr>
<tr>
<td></td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.45 0.20</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>1.50 0.20</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>1.50 0.20</td>
<td>3.4</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The incubation conditions and buffer used were as described in Table 2. All reaction mixtures contained 89 mg post-mitochondrial supernatant protein from dormant cysts plus substrate and cofactors as indicated.
glycolytic intermediates and subsequently to lactic acid. Collectively, these results indicate that glucose utilization (via glycolysis) by brine shrimp cyst post-mitochondrial fraction requires the addition of ATP and NAD\(^+\) for lactic acid production.

The Presence of Pyridine Nucleotides in Encysted Embryos and Nauplii of Artemia.

The presence of pyridine nucleotides in dormant cysts of *Artemia* was reported previously (Warner and Finamore, 1965), although the concentration of these nucleotides was not investigated. In the present study these cofactors were found to be present in very low levels in *Artemia* cysts and undetectable by TLC, paper chromatography or direct chemical analysis. Therefore, the amount of total NAD in cysts (determined as NAD\(^+\)) was estimated using the alcohol dehydrogenase (ADH) assay of Ciotti and Kaplan (1963), after extraction of 10 grams of dry cysts with cold acid, and purification of the NAD fraction on DEAE-cellulose (Warner and Finamore, 1967). The level of total NAD in nauplii was also determined by this procedure. The results of this study are shown in Table 7, and indicate that the level of NAD in dormant *Artemia* cysts is extremely low compared to nauplii. In fact, the level in the cysts is somewhat below the level of detection of the ADH assay. These values represent total NAD (NAD\(^+\) plus NADH) since the reduced form of the pyridine nucleotide was oxidized by acid during the extraction and purification of the pyridine nucleotides. Therefore, the relative amount of NADH in *Artemia* cysts and nauplii post-mitochondrial fraction was monitored indirectly using endogenous LDH which requires NADH as a cofactor in the conversion of pyruvate to
Table 7. The level of NAD⁺ in cysts and nauplii of *Artemia salina*.\(^a\)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>NAD⁺ (nmol/100,000 cysts or nauplii)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dormant cysts</td>
<td>&lt; 1.5</td>
</tr>
<tr>
<td>Nauplii</td>
<td>120</td>
</tr>
</tbody>
</table>

\(^a\)The amount of NAD⁺ was determined on partially purified acid extracts using the ADH procedure as described in Materials and Methods.
lactate. The results of this study are shown in Figures 5 and 6. The
data in Figure 5 indicate that pyruvate addition to a cyst post-
mitochondrial fraction had no effect upon lactic acid production,
whereas NADH addition stimulated lactic acid production. When pyruvate
and NADH were added together still greater quantities of lactic acid
were produced. The results in Figure 6 indicate that pyruvate addition
to a nauplii post-mitochondrial fraction had a substantial stimulatory
effect on lactic acid production indicating the presence of NADH in
the nauplii post-mitochondrial fraction. Although LDH is more active
in nauplii than in cysts as previously reported—(Ewing and Clegg, 1977)—,
it is clear that nauplii contain considerably more NADH than do cysts.
Furthermore, these data are consistent with the previous findings on
the level of total NAD in cysts and nauplii. Therefore, it appears
that the low concentration of pyridine nucleotides in dormant Artemia
cysts is responsible, at least in part, for the low level of lactic
acid produced in Artemia dormant cysts maintained under anoxia for
long periods of time.
Figure 4

Lactic acid production in post-mitochondrial fraction of Artemia cysts.

All reaction mixtures contained 178 mg of post-mitochondrial supernatant protein from dormant cysts in Buffer A plus no additions (●—●), 3.2 mM pyruvate (●—●), 0.67 mM NADH (●—●), 3.2 mM pyruvate and 0.67 mM NADH (●—●) in a final volume of 50 ml.

All reaction vessels were incubated at 30°C and aliquots were removed for lactic acid production at the times indicated.
Figure 5

Lactic acid production in post-mitochondrial fraction of *Artemia* nauplii.

All reaction mixtures contained 29 mg of post-mitochondrial supernatant protein from 21-hour nauplii in Buffer A plus no additions (●——●), 1.7 mM pyruvate (●—●), 0.36 mM NADH (●●●●●), 1.7 mM pyruvate and 0.36 mM NADH (●—●●), in a final volume of 25 ml. All reaction vessels were incubated at 30°C and aliquots were removed for lactic acid determination at the times indicated.
DISCUSSION

Previous studies on encysted embryos of the brine shrimp, *Artemia salina*, have indicated that carbohydrate metabolism is blocked when these embryos are maintained fully hydrated in a nitrogen environment (Dutrieu and Chrestia-Blanchine, 1966; Ewing and Clegg, 1969). In contrast, nucleotide metabolism continues at least to some extent under anoxia (Stocco et al., 1972). The reason for the block in carbohydrate metabolism may be severalfold. The cysts may be deficient in utilizable carbohydrates, contain a paucity of one or more glycolytic enzymes, lack sufficient ATP and/or NAD to permit glycolysis to proceed, or contain an inhibitor(s) of glycolysis. Whatever the mechanism that controls carbohydrate metabolism in *Artemia* cysts it appears to be an important adaptation for these embryos since they are often subjected to periods of anoxia in their natural environment (Green, 1961). The present investigation was undertaken in an attempt to elucidate the mechanism of control of carbohydrate metabolism in brine shrimp cysts exposed to varying periods of anoxia. Towards this objective, dormant encysted embryos of *Artemia salina* were examined for the presence of glycolytic enzymes by measuring the ability of various cyst homogenate preparations to produce lactic acid upon the addition of appropriate glycolytic substrates and/or cofactors. Furthermore, the importance of nucleotide cofactors in the glycolytic pathway and the presence of glycolytic inhibitor(s) in these cysts was also studied. The results of these experiments are discussed in relation to the ability of *Artemia salina* cysts to terminate carbohydrate metabolism under anoxia.
Contrary to the findings of previous investigators (Dutrieu and Christia-Blanchine, 1966; Ewing and Clegg, 1969) the present investigation demonstrated that encysted Artemia salina embryos accumulate some lactic acid when they are stored at room temperature in sterile ASW under nitrogen for various periods of time. In this study the cysts increased their lactic acid content two- to three-fold within twenty-four hours of anoxia and maintained this level for three months (Figures 1 and 2). Moreover, the increase in lactic acid production during anoxia was first observed at about the time the nauplius larva (in air) emerges from the chitinous shell (Figure 2). These results differ from those of Dutrieu and Christia-Blanchine (1966) who reported that "trace" quantities of lactic acid remain in the cysts after two months incubation under nitrogen at room temperature. Unfortunately, these authors neither indicated the method used for lactic acid measurements nor the value obtained.

Ewing and Clegg (1969) reported that Artemia salina cysts contain 2-3 µg lactic acid per mg protein and that this level remains constant for up to eight hours incubation under nitrogen. Although Ewing and Clegg used the same procedure for lactic acid determination as used in this study, their values are lower than those reported in this study because they did not delipidate their samples before measuring the protein content by the procedure of Lowry et al. (1951). We have found that lipid in Artemia salina embryo protein fractions interferes with the Lowry determination giving a higher than normal protein value and thus reduces the calculated amount of lactic acid/mg protein in the cyst (unpublished data). Also, the investigators carried out
their anoxia study for only eight hours, a period within which both aerated and nitrogen-treated cysts display similar lactic acid patterns (Figure 2). The results presented in this thesis parallel the LDH activity data of Ewing and Clegg (1969) who found that this enzyme increases after emergence (in air) and that lactic acid production in prenauplii and nauplii exposed to one hour of nitrogen is enhanced markedly. Since the viability of embryos in our batch of cysts was low (circa 30%) it was thought that the rise in lactic acid in cysts maintained under anoxia could be attributed to the non-viable cysts in the population. Therefore, the non-viable cysts were collected as previously described (Stocco et al., 1972), incubated in ASW under nitrogen at 30°C, then tested for lactic acid production at various times up to twenty-four hours. Although this lactic acid level was elevated (8.6 ± 1.0 μg lactic acid/mg cyst protein) in these cysts, it did not rise appreciably with time of anoxia. The lactic acid data of the present study are in disagreement with previous reports, but they may reflect a particular property of the batch of cysts tested. Nevertheless, the findings that the lactic acid level remains low in Artemia salina cysts and that development ceases in response to prolonged periods of anoxia are confirmed.

The observation that some lactic acid was produced under nitrogen indicates that the glycolytic enzymes are present in the dormant cysts. Indeed the addition of appropriate glycolytic intermediates and cofactors to reaction vessels containing cyst homogenates induced the production of some lactic acid in most cases. Only dihydroxyacetone phosphate, glyceraldehyde-3-phosphate and
glycerophosphate failed to elicit a response (Table 1). In general, the addition of glycolytic intermediates alone or cofactors alone (Table 2) (except for NADH) to homogenates of *Artemia* cysts did not significantly stimulate lactic acid production. Fructose-1,6-diphosphate alone appeared to stimulate markedly lactic acid production but since the products of the aldolase reaction (dihydroxyacetone phosphate and glyceraldehyde-3-phosphate) interfere with the lactic acid assay the meaning of these data remains obscure. The addition of NADH alone to cyst homogenates stimulated lactic acid production perhaps by converting endogenous pyruvate to lactic acid. Apart from these two observations it appears that the addition of both substrates and cofactors are necessary for the production of lactic acid by *A. salina* cyst homogenates. These results suggest that the cysts are deficient in glycolytic intermediates and/or cofactors, rather than glycolytic enzymes, and that this deficiency may be responsible for the low rate of lactate production by hydrated *A. salina* cysts maintained under nitrogen.

It appears certain that the lack of oxidizable substrates is not the cause of the problem. The major carbohydrates found in the encysted brine shrimp embryos are glycerol, glycogen, and trehalose (Dutrieu, 1960; Clegg, 1962; 1965; Dutrieu et al., 1966; Clegg, 1967). Of these carbohydrates, trehalose is the most abundant and comprises about 15% of the dry weight of the cyst (Clegg, 1965; Dutrieu et al., 1966). Trehalose is not used during dormancy since the concentration of this sugar is quite similar in cysts ranging in age from one to twenty-eight years in air (Clegg, 1962). However, when the dormant embryo is rehydrated under appropriate conditions and incubated in
air its trehalose level immediately begins to fall, decreasing essentially to "zero" by the time the nauplius is fully formed (Dutrieu, 1960; Clegg, 1964). Studies by Clegg (1964) also showed that in the dormant embryo, trehalose is the respiratory substrate and most of it is converted to glycogen and glycerol while the remainder is oxidized. After two months of anoxia, however, encysted embryos of *Artemia salina* were reported to increase slightly their level of trehalose and glycerol and to decrease slightly their content of glycogen (Dutrieu and Chrestia-Blanchine, 1966).

Since trehalase is important in the mobilization of trehalose, and therefore in development, the activity of this enzyme was measured in *Artemia salina* cyst homogenates and in a crude enzyme fraction (CEF). The addition of trehalose to cyst homogenates (which include considerable endogenous trehalose) failed to stimulate greatly the production of lactic acid even in the presence of cofactors (Table 1). However, alcohol precipitation of CEF doubled the activity of trehalase compared to untreated CEF (Table 4). Interestingly enough, Friedman (1960) purified trehalase from the adult insect *Phormia regina* using ethanol (40-60%) to precipitate the enzyme from the post-mitochondrial fraction (PMF) (see also Hill and Sussman, 1963). This observation is interesting and suggests that trehalase in *Artemia salina* cysts may be non-functional until activated in some way shortly after resumption of development. Furthermore, it was found that high concentrations of trehalose (greater than 1.6 mM) were required to demonstrate activity with either untreated or alcohol-treated CEF. Taken together, these results indicate that trehalase may be an important enzyme in the
control of carbohydrate metabolism in *Artemia salina* cysts. However, the activation of trehalase may be directly linked to the overall metabolic state of the embryo and to the availability of trehalose rather than to the absence or presence of oxygen in the environment.

Since some glycolytic enzymes displayed low activity in cyst homogenates after the addition of appropriate glycolytic intermediates and cofactors, the activity of some of these enzymes was also tested using a crude enzyme fraction (CEF) devoid of small molecular weight components (Table 5). Hexokinase, phosphohexoseisomerase, phosphofructokinase, and aldolase were all found to be active in the CEF, but it appeared that hexokinase and phosphofructokinase in the CEF require high concentrations of substrate and cofactor(s) to stimulate lactic acid production. These results support the idea that these glycolytic enzymes are present in *A. salina* embryos and suggest that hexokinase and phosphofructokinase may be rate limiting in *Artemia salina* cysts.

While most of the glycolytic enzymes appeared to be active in the cysts, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, and glycerophosphate dehydrogenase appeared to be inactive in the production of lactic acid. Therefore, the activity of these enzymes was determined indirectly by monitoring the appearance (or disappearance) of NADH (at 340 nm) after the addition of cyst post mitochondrial fraction (PMF) to a reaction mixture containing either glyceraldehyde-3-phosphate or dihydroxyacetone phosphate and NADH or NAD⁺. The results of this study demonstrated that the addition of either glycolytic intermediate produced a rapid disappearance of NADH. On the other hand, the addition of glyceraldehyde-3-phosphate to
a reaction mixture containing dialyzed PMF and NAD\textsuperscript{+} did not cause a measurable increase in absorbance at 340 nm. These results suggest that triosephosphate isomerase and glycerophosphate dehydrogenase are present in \textit{A. salina} cysts. The presence of glyceraldehyde-3-phosphate dehydrogenase, however, is uncertain since any NADH produced in the reaction catalyzed by this enzyme would be rapidly re-oxidized to NAD\textsuperscript{+} by glycerophosphate dehydrogenase in the PMF or CEF. Furthermore, these results suggest that the glycolytic pathway in \textit{A. salina} cysts may greatly favor the production of glycerol rather than lactic acid. Indeed, the equilibrium constants of these enzymes support this hypothesis. The $K_{eq}$ of glyceraldehyde-3-phosphate dehydrogenase is that of triosephosphate isomerase is 22, and that of glycerophosphate dehydrogenase is $10^{12}$ (Barman, 1969). Clegg (1964) suggested that glycerol in \textit{A. salina} embryos is synthesized through an enzymic reduction of dihydroxyacetone phosphate as demonstrated for certain diapausing insects where free glycerol also accumulates. These results may also explain the observed "lactic acid" value obtained when dihydroxyacetone phosphate was added to cyst homogenate (Table 1). In the formation of glycerol, the $K_{eq}$ of the glycerophosphate dehydrogenase reaction is considerably greater than the $K_{eq}$ of triosephosphate isomerase reaction leading to lactate. Therefore, dihydroxyacetone phosphate should be converted readily to glycerophosphate (in the presence of NADH) and the color interference reduced. Thus, it appears that the enzymes triosephosphate isomerase and glycerophosphate dehydrogenase in \textit{A. salina} embryos are active in the production of glycerol rather than lactic acid, whereas glyceraldehyde-3-phosphate dehydrogenase appears to be relatively inactive.
The possibility that the small molecular weight components in
cysts may have affected the production of lactic acid by
homogenates was also examined. The addition of these components (as a
cytosol dialyze fraction) to reaction mixtures containing CEF and
either glucose or fru-6-P and the necessary cofactors, stimulated the
formation of lactic acid (Figure 3). Therefore, glycolysis in _A.
salina_ embryos does not seem to be inhibited by any component found in
the dialyze or small molecular weight fraction.

Zotin (1972) reported that the rate of glycolysis can be modified
by the rates of coupled processes such as gluconeogenesis, hexose
monophosphate shunt (HMP-S) or Krebs cycle. These processes can compete
with glycolysis as well as with each other for substrates and cofactors
such as NAD or ATP. The idea that gluconeogenesis may modify the rate
of glycolysis or carbohydrate metabolism in _A. salina_ cysts under
anoxia appears to have no basis. Most of the carbohydrate in the cysts
is already in a storage form (trehalose and glycogen) and is utilized
during embryogenesis. However, Dutrieu et al. (1966) reported a rise
in the level of trehalose in _A. salina_ cysts incubated for two months
under anoxia. Since trehalose is converted to glycogen and glycerol,
as well as CO₂, during normal development (Clegg, 1965), it appears
that trehalose synthesis in the absence of oxygen may be the result of
shutdown of carbohydrate utilization rather than the cause. The HMP-S
is generally recognized as playing an extremely important role during
respiration of sea urchin embryos (Stearns et al., 1974). However, the
presence of this pathway in _A. salina_ embryos has not been studied
and therefore, its effect upon carbohydrate metabolism under either
aerobic or anaerobic conditions is unknown. The competition between the Krebs cycle and glycolysis in *A. salina* cysts in the absence of oxygen should theoretically be non-existent. However, mitochondria have been reported to excrete two factors into the ambient medium of a cell-free system that stimulate glycolysis (Milman and Yurowitzy, 1969). One of these factors has been identified as nucleotide cofactors: ATP, ADP, and trace amounts of NAD. The second is the lipoprotein factor, "kinasine" which controls the activity of phosphoglycerate kinase (see also Zotin, 1972). The data obtained thus far suggest that carbohydrate utilization in *Artemia* embryos under anoxia is controlled by a mechanism(s) other than those described above.

Zotin (1972) has provided an excellent review of the regulation of glycolysis in embryonic development based on the work of Milman and Yurowitzky on the loach embryos and on fish eggs. In his view, regulation of glycolysis in embryonic development can be determined by mechanisms similar to those which are responsible for the regulation of respiration: change in the level of enzymes, substrates and cofactors, and conformation of intracellular membranes. However, Zotin stresses that of the possible mechanisms responsible for the regulation of glycolysis in embryos, the change in rate of glycolysis during development is not determined by the amount of enzymes but by changes in their activity as a result of various regulatory mechanisms.

In the present study, the homogenates of *Artemia* cysts were found to contain all the glycolytic enzymes necessary for the production of lactic acid (with the possible exception of glyceraldehyde-3-phosphate dehydrogenase). However, the addition of both glycolytic intermediates and cofactors were essential for the formation of lactic acid by
these homogenates (Table 1). Since dormant *A. salina* cysts do not appear to contain an inhibitor of glycolysis and contain a large source of trehalose for the production of glycolytic intermediates, the only other obvious possibility for control of carbohydrate metabolism in these cysts appears to be the level of cofactors. Indeed the results of Table 6 support this hypothesis. The addition of glucose alone to concentrated PMF of *A. salina* cysts did not stimulate the production of lactic acid until ATP and/or NAD$^+$ were added to the reaction mixture.

It was previously reported that the cofactors ATP and NAD$^+$ existed in very low concentrations in dormant *A. salina* cysts (Warner and Finamore, 1965; 1967; Stocco et al., 1972). Stocco et al. (1972) found that *A. salina* cysts stored under nitrogen continued nucleotide metabolism and produced marked changes in the adenosine nucleotide level. Their results showed that the concentration of ADP and ATP decreased to undetectable levels in cysts exposed to nitrogen. Although the rate of disappearance of these nucleotides was variable and batch dependent under anoxia, aeration of these embryos restored ADP and ATP to levels found in control embryos at the time of hatching. Similar observations were made by other investigators working with frog eggs placed under anoxia (Barth and Barth, 1954; Brachet, 1960), and Barnett (1953) found that exogenously added ATP could reverse the inhibition of division by anaerobiosis in sea urchin eggs. Thus, it appears that development ceases or is arrested, as in the case of *A. salina* when embryos are subjected to anaerobiosis primarily because of a diminution in ATP to concentrations below the "critical" level required for normal developmental processes.

The pyridine nucleotides (NAD$^+$ and NADH) are also essential for
glycolysis and in brine shrimp embryos these nucleotides may be of primary importance in the control of glycolysis during anoxia. These nucleotides are required in the following reactions:

1. glyceraldehyde-3-phosphate + NAD$^+$ \[\xrightarrow{\text{dehydrogenase}}\] 1,3-diphosphoglyceric acid + NADH + H$^+$

2. dihydroxyacetone phosphate + NADH + H$^+$ \[\xrightarrow{\text{dehydrogenase}}\] L-$\alpha$-glycerophosphate + NAD$^+$

3. pyruvic acid + NADH + H$^+$ \[\xrightarrow{\text{lactic dehydrogenase}}\] lactic acid + NAD$^+$

From reactions (2) and (3) above, it is clear that glycerophosphate dehydrogenase and LDH may compete for NADH and this has been shown to occur in the flight muscle of insects (Gilmour, 1961; 1965). Since glyceraldehyde-3-phosphate dehydrogenase (1) is important for the regeneration of NADH, this enzyme may be the most important one in *A. salina* for the production of cytoplasmic NADH unless other means are available to generate NADH. Although the presence of NAD in Artemia cysts was reported by Warner and Finamore (1965), all attempts to measure the level of NAD$^+$ (or NADH) in cysts were unsuccessful (Figure 4, Table 7). In contrast, 21-hour nauplii contain NAD$^+$ and NADH in large amounts compared to the cysts (Figure 5). Therefore, it appears that the pyridine nucleotides are limiting in dormant cysts and therefore important as regulators of carbohydrate metabolism in embryos exposed to anoxia.

Another mechanism which has been implicated recently in the control of lactic acid production in helminths and bivalves is the
amino acid alanine (Harpur, 1974; Mustafa, 1974). Under anaerobic conditions pyruvic kinase is inhibited by decreasing pH and alanine which accumulate along with succinate (Harpur, 1974). Whether succinate is produced in A. salina cysts during development has not been studied, but the level of alanine in developing cysts has been investigated by Emerson (1967). Soon after resumption of development (in air) alanine rises rapidly, reaches a peak shortly before emergence, then falls rapidly concomitant with a rise in oxygen consumption and the appearance of the prenauplius larva. Since the greatest rise in lactic acid occurs at about the time of emergence when the alanine concentration is greatest, it appears that alanine is not of major importance in blocking glycolysis. However, we have no data on the alanine levels of cysts under anoxia and thus it is difficult to assess the potential importance of this amino acid in the regulation of pyruvate formation. It would be interesting to determine whether the alanine profile of A. salina cysts incubated under nitrogen mimics that of aerobic development.

The present study was undertaken to elucidate the mechanism responsible for the termination of carbohydrate metabolism in A. salina cysts placed under anoxia. Contrary to previous findings, some lactic acid accumulates in these cysts under anoxia indicating that some carbohydrate metabolism occurs within twenty-four hours under anoxia. This finding is in agreement with the results of Stocco et al. (1972) who found that nucleotide metabolism continues in embryos that are incubated under nitrogen for various periods of time. Moreover, this study may also provide some basis for the observations of Dutrieu et al. (1966) who reported that the levels of trehalose and
glycerol increase in cysts maintained under anoxia for two months. the idea that development does not stop abruptly in embryos exposed to anaerobiosis is consistent with previously studied embryonic systems. Still, carbohydrate metabolism, as measured in terms of lactic acid production, stops after one day exposure to nitrogen.

The findings of this study may explain why hydrated encysted embryos of *A. salina* do not accumulate large amounts of lactic acid in the absence of oxygen. Dormant cysts initially contain limited supplies of both ATP and NAD. Thus, the exposure of these embryos to optimal conditions in the absence of oxygen allows some development to occur. However, the high energy demand for development, and especially glycerol production (for emergence) depletes ATP (and perhaps NADH) preventing any further breakdown of trehalose and/or glycogen via glycolysis. Since the synthesis of glycerol from trehalose probably requires energy, unless other means are available, it would appear that the oxidation of trehalose via glycolysis is the major source of energy for development. Thus, any mechanism which decreases the supply of energy will also slow development and therefore the time required for emergence to occur (see Clegg, 1964). It is interesting to note that of all the glycolytic enzymes studied in dormant cyst homogenates, the three enzymes which appeared to be inactive in lactic acid formation (triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase) are at the crossroad between glycerol formation and lactic acid formation. Moreover, since triosephosphate isomerase and glyceraldehyde dehydrogenase appear to favor the production of
glycerol, whereas glyceraldehyde-3-phosphate dehydrogenase appeared to be inactive, it seems that glycolysis in *A. salina* embryos is programmed towards glycerol formation for the process of emergence of the prenauplius. Since glycerol formation requires ATP and NADH, the production of lactic acid becomes of secondary importance to the cysts. Consequently, ATP and NADH as well as the triose phosphates are no longer available in adequate amounts for the production of additional ATP to replenish the supply used for glycerol formation. Subsequently, the ATP content of the cysts is depleted and the oxidation of trehalose and glycogen comes to a standstill.

Since emergence is critical to survival, encysted embryos of *Artemia salina* have elected to utilize glycolysis mainly for glycerol formation. Thus, in the absence of an adequate supply of oxygen, *Artemia salina* cysts, in their attempt to emerge, deplete their low levels of ATP and NAD in the production of glycerol, preventing further utilization of the carbohydrate stores and accumulation of lactic acid. Unless the need for ATP (and NAD) in encysted embryos of *Artemia salina* is readily met by oxidative metabolism (respiration), development (morphogenesis and emergence) stops.
SUMMARY

1. During three months of anoxia, encysted embryos of Artemia salina synthesize only a small amount of lactic acid (from 4.6 to 11.6 μg/mg cyst protein) and this increase occurs within the first day.

2. The increase in lactic acid content in cysts under anoxia occurs at about the time that control embryos (in air) begin to emerge.

3. The most active enzymes in the production of lactic acid by cyst homogenates are aldolase, pyruvic kinase, and LDH. All glycolytic enzymes appear to be present in encysted embryos of Artemia salina except, perhaps, for glyceraldehyde-3-phosphate dehydrogenase.

4. Trehalase is also present in cysts and its activity can be increased twofold by treating a cyst crude enzyme preparation with 60% ethanol.

5. The production of lactic acid by cyst homogenates from glucose requires the addition of ATP and NAD⁺.

6. NAD appears to be present in limiting amounts in Artemia salina cysts.

7. Artemia salina cysts appear to terminate carbohydrate metabolism under anoxia by depleting their ATP stores and because of a limiting supply of NAD.
LITERATURE CITED


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